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Chimerasome-mediated gene transfer in vitro and in vivo

(Recombinant DNA; transfection; liposomes; proteoliposomes; cochleates; phalloidin; drug delivery; microinjection; membrane fusion; glycoproteins)

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SUMMARY

Proteoliposome delivery vesicles can be prepared by the protein-cochleate method [Gould-Fogerite and Mannino, Anal. Biochem. 148 (1985) 15–25; Mannino and Gould-Fogerite, Biotechniques 6 (1988) 682–690]. Proteins which mediate the entry of enveloped viruses into cells are integrated in the lipid bilayer, and materials are encapsulated at high efficiency within the aqueous interior of these vesicles. We describe proteoliposome-mediated delivery of proteins and drugs into entire populations of cells in culture. Material can be delivered gradually by Sendai-virus-glycoprotein-containing proteoliposomes. Alternatively, synchronous delivery to a population can be achieved by exposing cell-bound influenza glycoprotein vesicles briefly to low pH buffer. When DNA is encapsulated, chimeric proteoliposome gene-transfer vesicles (chimerasomes), which mediate high-efficiency gene transfer in vitro and in vivo, are produced. Stable expression of a bovine papilloma virus-based plasmid in tissue-cultured cells, at 100 000 times greater efficiency than Ca · phosphate precipitation of DNA, with respect to the quantity of DNA used, has been achieved. Stable gene transfer and expression in mice has been obtained by subcutaneous injection of chimerasomes containing a plasmid expressing the early region of polyoma virus. In one experimental group, 50% of the mice developed tumors which were shown to express polyoma virus early proteins and contain the transferred DNA. This is the first report of stable gene transfer in animals mediated by a liposome- or proteoliposome-based system.

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Abbreviations: bp, base pair(s); BPV-1, bovine papilloma virus type 1; BSA; bovine serum albumin; DC method, see MATERIALS AND METHODS, section **a**; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HA, hemagglutinin glycoprotein of influenza virus; Ig, immunoglobulin; IgG-LY, Ig class G-Lucifer Yellow complex; kb, kilobase(s) or 1000 bp; LC method, see MATERIALS AND METHODS section **a**; large T, middle T, and small T, tumor antigens of PyV; NA, neuraminidase glycoprotein of influenza virus; nt, nucleotide(s); *ori*, origin of DNA replication; PyV, polyoma virus; TES buffer, see MATERIALS AND METHODS section **a**; TES/MES buffer, see legend to Fig. 2.

INTRODUCTION

A complete understanding of many complex biological processes requires investigation within the context of living cells or animals. One of the fundamental and most powerful methods of biological research is to perturb living systems and to observe the consequences. This approach usually relies on, and is often limited by, the ability to introduce drugs and other small molecules, proteins, or nucleic acids into living cells or animals. Transiently changing the biochemical state or permanently changing the genotype, and consequently phenotype, of a cell or animal through the introduction of exogenous DNA can yield information not readily obtainable by other methods (Szybalska and Szybalski, 1962).

Enveloped viruses have evolved to be highly efficient gene transfer vehicles. These viruses are composed of a nucleic acid and protein core surrounded by a lipid bilayer that contains integral membrane proteins which project outwards. These membrane glycoproteins mediate viral entry into animal cells by binding to receptors on the plasma membrane and bringing about the fusion of the viral envelope with the cellular membrane (reviewed in Dimmock, 1982). This fusion occurs either at the surface of the cell or within the low pH environment of an endocytic vesicle (reviewed in White et al., 1983) and results in delivery of the viral core into the cytoplasm.

A number of different laboratories have utilized proteoliposomes containing reconstituted viral envelope proteins, for delivery of molecules to animal cells (Uchida et al., 1979; Vainstein et al., 1983; Volsky et al., 1984; Kaneda et al., 1987; 1989; Tikchonenko et al., 1988). In contrast to previously reported procedures for reconstituting biologically active membrane proteins into liposomes (reviewed in Racker, 1985), the protein-cochleate technique allows for the high-efficiency encapsulation of molecules within the aqueous interior of large (0.1 to 4.0 μ diameter) unilamellar vesicles (Gould-Fogerite and Mannino, 1985; Gould-Fogerite et al., 1988). In addition, organic solvents, sonication, chaotropic ions, or extremes of temperature, pH or pressure are not required for the production of these vesicles. Thus, the physical integrity and biological activity of proteins integrated in the lipid bilayer and materials encapsulated in the aqueous interior are maintained. We report here that these Sendai and influenza delivery vesicles can be used to deliver proteins and drugs to entire populations of cells in culture.

'Chimerasomes', modified reconstituted viral envelopes containing encapsulated DNA, are prepared by the protein-cochleate method described in this report. These structures can mediate high-efficiency transient and stable gene transfer to cells in culture. Several methods have been employed to achieve gene transfer in mammals, including direct injection of naked or Ca·phosphate-precipitated DNA (Asselin et al., 1983; Dubenski, 1984; Benvenisty and Reshef, 1986), infection with virus (Desrosiers et al., 1985), transplantation of transformed or infected cells (see e.g., Cline et al., 1980; Anderson, 1984; Eglitis et al., 1985; Dzierzak et al., 1988; Stead et al., 1988; St. Louis and Verma, 1988), and production of transgenic animals (reviewed in Palmiter and Brinster, 1985; Camper, 1987). A number of laboratories are investigating liposome-mediated gene transfer (Wong et al., 1980; Straubinger and Papahadjopoulos, 1983; Vainstein et al., 1983; Itani et al., 1987; Nicolau et al., 1987; Wang and Huang, 1987; Mannino and Gould-Fogerite, 1988; Kaneda et al., 1989). This is the first report of the use of a liposome- or proteoliposome-based system to achieve stable gene transfer and expression in animals.

MATERIALS AND METHODS

(a) Preparation of proteoliposome delivery vesicles and chimerasomes

The protein-cochleate method of proteoliposome formation has been previously described in detail (Gould-Fogerite and Mannino, 1985). Briefly: Sendai and influenza viruses were grown in embryonated chicken eggs and purified by differential centrifugation. The viral envelope was extracted at 37°C at a concentration of 2 mg viral protein/ml with 2% (w/v) *n*-octyl-β-D-glucopyranoside in 2 M NaCl/0.02 M Na·phosphate buffer pH 7.4. Viral cores were removed by centrifugation (60 000 × *g*, 5°C, 60 min). The solubilized viral envelope was added to a film of phosphatidylserine and cholesterol (9 : 1 w/w) equal to four times the weight of the viral glycoproteins (which comprise one-third of the total

protein of the virus), and then vortexed. This solution was then dialysed against TES buffer (2 mM *N*-Tris[hydroxymethyl]-methyl-2 aminoethane sulfonic acid/2 mM histidine/100 mM NaCl pH 7.4) to form small proteoliposomes, then dialysed against TES buffer with 3 mM $CaCl_2$ to form a protein-cochleate precipitate (LC method: liposomes before cochleates). Alternatively, the viral envelope-phosphatidylserine-cholesterol solution was dialyzed against TES buffer with 3 mM $CaCl_2$, to form protein-cochleates in one step (DC method: direct calcium dialysis cochleates). Cochleates were pelleted (60 000 × *g*, 5 °C, 60 min), the supernatant removed, and replaced with a small volume of TES buffer containing the material to be encapsulated. Samples to be encapsulated contained protein concentrations which ranged from 1 to 10 mg/ml and DNA was 1 mg/ml. Cochleates can be stored for several months under N_2 in tightly sealed tubes at 4 °C, after pelleting and resuspension in TES/3 mM $CaCl_2$. Proteoliposomes are formed from cochleates by chelation with EDTA of the Ca^{2+} contained in the cochleate complex. This can be accomplished by adding small aliquots of 150-mM EDTA pH 9.5, followed by 150-mM EDTA pH 7.4 (with monitoring to maintain pH near neutral), directly to a suspension of cochleates and the material to be encapsulated. Alternatively, rotary dialysis overnight against TES buffer with 10 mM EDTA may be used (see Gould-Fogerite and Mannino, 1985, for description of rotary dialysis). Direct addition of EDTA gives rapid formation of vesicles and encapsulation of large and small molecules at efficiencies up to 15% of added materials. Rotary dialysis gives a higher quantity and quality reconstitution of the viral glycoproteins, higher encapsulation efficiencies (up to 40%), and is particularly useful for very large molecules. Large vesicles were separated from small vesicles, nonintegrated protein, and nonencapsulated material by column chromatography using Sephacryl S-1000 (Gould-Fogerite et al., 1988).

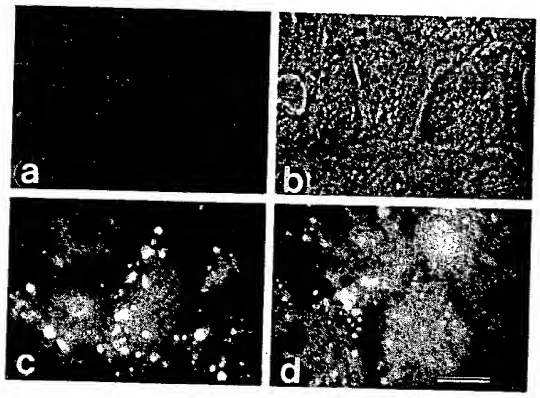

Fig. 1. Sendai proteoliposome delivery of IgG-LY to rat PTK_1 cells. (Panel **a**) Fluorescence micrograph of cells incubated with free IgG-LY alone. (Panel **b**) Phase-contrast micrograph of the same field. Untreated cells or cells incubated with buffer-filled Sendai proteoliposomes, alone or with nonencapsulated IgG-LY, are not shown, but were similarly nonfluorescent. (Panels **c** and **d**) Fluorescence micrographs of representative fields after 18 h incubation with IgG-LY-loaded Sendai vesicles. All photomicrographs are of live unfixed cells, using the same exposure and developing conditions. Goat anti-rat IgG was covalently labeled with LY and separated from free LY by column chromatography on G25 Sephadex (Stewart, 1981). IgG-LY was encapsulated within proteoliposomes containing Sendai virus glycoproteins integrated in the lipid bilayer, prepared by the DC method, using rotary dialysis at the vesicle formation stage (Gould-Fogerite and Mannino, 1985). Large IgG-LY-filled liposomes were isolated from nonencapsulated and nonintegrated material, by chromatography on Sephacryl S-1000 (Gould-Fogerite and Mannino, 1985; Gould-Fogerite et al., 1988). These proteoliposomes were incubated with rat PTK_1 cells in low Ca^{2+} medium with 10% FBS at 37 °C, 5% CO_2 on glass coverslips in 24-well cluster plates. DMEM specially made to be 0.5 mM Ca^{2+} and Mg^{2+}-free was used for all liposome-cell incubations, because the high concentration of Ca^{2+} in DMEM tends to destabilize liposomes made from negatively-charged phospholipids (Papahadjopoulos et al., 1975; Mannino and Gould-Fogerite, 1988). Coverslips were removed from the well, washed with TES buffer by dipping and draining to remove nonadherent vesicles, and examined by fluorescence microscopy after 18 h.

RESULTS AND DISCUSSION

(a) Antibodies can be delivered into the cytoplasm of an entire population of living cells in culture

Proteoliposomes containing Sendai virus glycoproteins integrated in the lipid bilayer were used to deliver antibodies to the cytoplasm of cells in culture (Fig. 1, panels **c** and **d**). IgG-LY (Stewart, 1981) was encapsulated in Sendai vesicles, and incubated with rat PTK1 fibroblasts for 2, 4, or 18 h. After removal of nonadherent vesicles, unfixed living cells were examined by fluorescence microscopy. After 2 or 4 h incubation, many cell-surface-adherent vesicles were

present, but little or no cytoplasmic fluorescence was seen. After 18 h incubation, cell-surface-adherent vesicles, as well as bright, diffuse, cytoplasmic fluorescence, was seen with virtually all cells of all fields (Fig. 1, panels **c** and **d**). The entire area of the cell, with the exception of large intracellular vacuoles, was filled with fluorescence (Fig. 1, panels **c** and **d**). This pattern of fluorescence distribution confirmed that the antibody had been delivered to the cytoplasm, rather than sequestered in surface-adherent or cytoplasmic vesicular structures. There was some variability from cell to cell in the intensity of fluorescence, probably reflecting different quantities of material delivered. Controls, including cells which were untreated, cells incubated with free IgG-LY (Fig. 1, panel **a**), buffer-filled proteoliposomes, or preformed, buffer-filled proteoliposomes mixed with free IgG-LY, were not fluorescent.

(b) Drugs, or other small molecules, can be synchronously delivered to a population of cells by brief exposure of cell-bound influenza vesicles to low pH

The ability of vesicles containing influenza virus glycoproteins (HA and NA) integrated in the lipid bilayer to deliver a drug to the cytoplasm of living cells was investigated. Phalloidin, a bicyclic peptide (M_r 1250) isolated from the toxic *Amanitus* mushroom, binds to filamentous actin, stabilizes it, and in some instances may promote its polymerization (Wulf et al., 1979). FITC-phalloidin has been used to localize cellular actin by reaction with fixed cells or by microinjection into living cells (Wehland et al., 1977; Hamaguchi, 1982). When FITC-phalloidin was encapsulated in influenza delivery vesicles and incubated with cells overnight at neutral pH, little or no cytoplasmic fluorescence was seen (Fig. 2, panels **a** and **b**). Exposure of influenza virus to low pH causes a conformational change in the HA glycoprotein, which is associated with, and may be necessary for, its ability to mediate membrane fusion (reviewed in White et al., 1983). Intracellular delivery to all the cells in the culture was achieved when influenza glycoprotein vesicles containing encapsulated FITC-phalloidin were incubated with cells overnight in medium at neutral pH, followed by exposure to pH 5.0 buffer for 1 min, and returned to medium at pH 7.4 at 37°C. At short times after low pH treatment, diffuse cytoplasmic fluorescence was

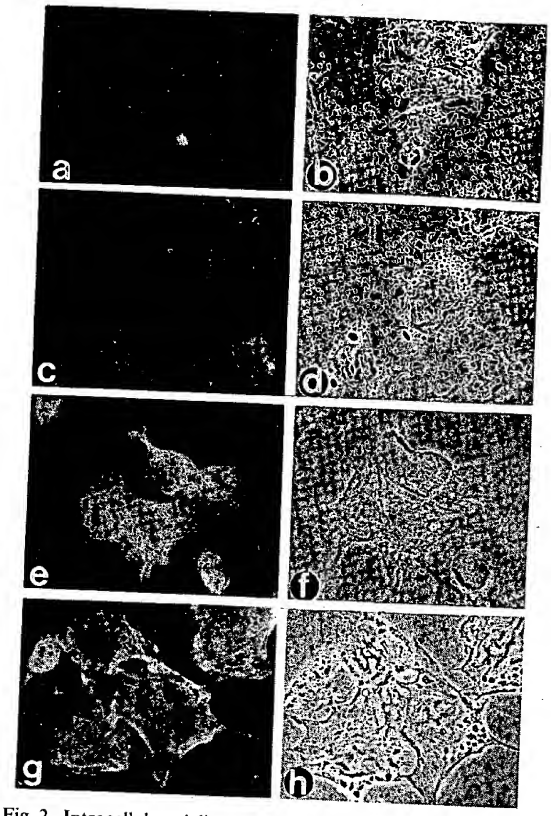

Fig. 2. Intracellular delivery and targeting of phalloidin by influenza proteoliposomes. (Panels **a** and **b**) Fluorescence and phase photomicrographs of C127 cells incubated overnight with buffer-filled influenza proteoliposomes and free fluorescein-phalloidin. (Panels **c**, **e** and **g**) fluorescence, (panels **d**, **f** and **h**) phase-contrast photomicrographs of cells incubated overnight with fluorescein-phalloidin-loaded influenza proteoliposomes. (Panels **c** and **d**) C127 cells 15 min after a 1.0-min exposure to pH 5.0 buffer to trigger vesicle-cell fusion. (Panels **e** and **f**) C127 cells 2.5 h after 1.0-min pH 5.0 treatment. (Panels **g** and **h**) rat LRM-55 glial cells 4 h post pH 5.0 treatment. Influenza vesicles made by the LC method and using direct addition of EDTA at the vesicle formation step (Gould-Fogerite and Mannino, 1985), with encapsulated fluorescein-labeled phalloidin, were incubated overnight with either mouse C127 mammary tumor-derived cells or rat LRM55 glial tumor cells. Glass coverslips containing cells and adherent vesicles were rinsed by dipping in TES buffer (pH 7.4), and draining by touching the edge to absorbent paper. Coverslips were dipped and drained, then submersed in TES/MES (100 mM NaCl/2 mM histidine/1 mM TES/1 mM 2[*N*-morpholino]ethanesulfonic acid pH 5.0) for 1 min to activate the influenza glycoprotein-mediated vesicle fusion. The cells were returned to neutrality by dipping in TES buffer (pH 7.4) and draining. After subsequent incubation in low-Ca^{2+} medium at 37°C, coverslips were rinsed in TES buffer pH 7.4 and examined by fluorescence microscopy.

seen (Fig. 2, panels **c** and **d**). Several hours after exposure of cell-bound vesicles to low pH, fluorescence appeared to be organized in a linear fashion, indicating binding to filamentous structures in the cytoplasm, presumably actin (Fig. 2, panels **e–h**). Diffuse cytoplasmic fluorescence was also seen. In living cells, in addition to large actin-containing structures, phalloidin binds to short actin polymers, and some phalloidin also remains free in the cytoplasm. In contrast, when *fixed* cells are stained with molecules targeted to such structural components, more defined patterns are seen, because the cells are permeabilized, and unbound material is removed by extensive washing.

The linear-patterned fluorescence seen when phalloidin was delivered to cells was in marked contrast to the purely diffuse fluorescence obtained when molecules without specific intracellular targets, such as nonspecific antibodies (Fig. 1), FITC-dextran, FITC-horseradish peroxidase, calcein, or carboxyfluorescein, have been used (not shown). In addition, the delivery of phalloidin was dependent on a brief low pH treatment of the cell-bound vesicle population. These and other data, not included here, strongly support the retention of low pH-activated membrane fusion activity of the influenza HA glycoprotein, when reconstituted using the protein-cochleate method. This is the first report of low-pH-activated delivery by liposomes containing reconstituted influenza glycoproteins.

Controls consisting of untreated cells, or cells incubated with free phalloidin alone and in combination with preformed buffer-filled vesicles, were nonfluorescent (e.g., Fig. 2, panel **a**). All photomicrographs in Figs. 1 and 2 are of live, unfixed cells, using the same photographic and developing conditions within each figure.

The ability to introduce biologically active drugs and proteins into the cytoplasm of living cells, and observe the consequences, should facilitate many studies which were previously dependent on microinjection, since large populations of altered cells can now be generated.

(c) High efficiency gene transfer in culture can be mediated by Sendai chimerasomes

We use the term 'chimerasome' to describe the structures which result when nucleic acids or nucleic acids and proteins are encapsulated within modified viral envelopes using the protein-cochleate method (Gould-Fogerite and Mannino, 1985; Mannino and Gould-Fogerite, 1988). These structures maintain the cellular attachment and fusion properties of the viruses from which the envelope originates. However, rather than the original infectious viral core, any nucleic acid or nucleic acid-protein complex of interest can be encapsulated within the aqueous interior of these modified envelopes. These structures can be used to deliver biologically active nucleic acids into the cytoplasm of target cells.

Sendai chimerasomes were utilized for gene transfer to cells in tissue culture. Stable expression of the transferred DNA was obtained (Table I). Plasmid pNS1-8, which contains the entire BPV-1 genome, plus plasmid sequences which allow amplification in *Escherichia coli*, is capable of stable replication as an episome within the nuclei of mouse mammary tumor-derived C127 cells (Sarver et al., 1982; Howley et al., 1983). Acquisition and stable expression of this plasmid causes these normally well-spread, contact-inhibited cells to become spindle-shaped and form foci within the monolayer of untransformed cells. Sendai chimerasomes containing this plasmid were incubated with C127 cells overnight. Alternatively, the plasmid was delivered to C127 cells using the Ca · phosphate coprecipitation method (Graham and Van der Eb, 1973). Maximum transfection using Ca · phosphate coprecipitation required 1 to 10 μg of plasmid DNA, and resulted in approx. 200 foci per 10^6 cells. These results are consistent with data previously reported by others for this plasmid and cell line (Sarver et al., 1982; Howley et al., 1983). A similar number of foci was generated by use of Sendai chimerasomes for transfection. The presence of BPV-1 DNA in the foci was confirmed by in situ hybridization with radiolabelled plasmid (Villarreal and Berg, 1977).

The efficiency of transfection using Sendai chimerasomes, however, was much less dependent on the concentration of DNA. Near-maximal transfection was achieved with approx. 2.3 pg of encapsulated DNA and 330 pg of phospholipid per 10^6 cells. This represents approx. 1.8×10^5 copies of the plasmid and 2×10^5 vesicles, presuming an average vesicle diameter of 1 μm (vesicles produced by this method range from 0.1–4 μm in diameter). In contrast, the ability to transfect using Ca · phosphate is

TABLE I

Stable gene transfer and expression in cells in culture mediated by Sendai chimerasomes[a]

	μg of DNA[d]	Number of foci[e]	Number of foci/μg DNA
Ca · phosphate coprecipitation[b]	10	250	25
	1	175	175
	0.1	23	230
	0.01	2	200
Sendai chimerasomes[c]	9.5	172	18
	2.37	143	60
	0.023	134	5.8×10^3
	0.00023	160	6.9×10^5
	0.0000023	91	3.9×10^7

[a] A BPV-1-based plasmid which replicates in the nucleus of cells, was delivered to cells within Sendai chimerasomes or by Ca · phosphate precipitation. Plasmid pNS1-8 contains the entire BPV-1 genome and *E. coli* plasmid pBR322 derivative, pML2d (same as p142.6 minus a *Bam*HI site; Howley et al., 1983; Sarver et al., 1982).

[b] C127 cells were plated at 5×10^5 cells/60-mm dish. 24 h later, DNA was delivered to cells by coprecipitation of naked DNA with Ca · phosphate (Graham and Van der Eb, 1977). After 72 h, cells were trypsinized and replated at lower density.

[c] Plasmid pNS1-8 was encapsulated in Sendai glycoprotein-containing liposomes made by the LC method and using rotary dialysis at the vesicle-formation step (Gould-Fogerite and Mannino, 1985), and then incubated with C127 cells overnight in low Ca^{2+} DMEM with 10% FBS. Cell culture was as described in section **b**.

[d] Liposome-encapsulated DNA was quantitated by radioactivity from nick-translated [^{32}P]plasmid, which had been included in a trace amount (Rigby et al., 1977).

[e] Acquisition and stable expression of pNS1-8 causes foci formation by these normally contact-inhibited cells. Macroscopically visible foci were counted at eight weeks. The presence of BPV-1 sequences in foci was confirmed by transfer to nitrocellulose and in situ hybridization with nick-translated [^{32}P]pNS1-8 (Villarreal and Berg, 1977).

rapidly lost when quantities less than the optimal 1–10 μg of plasmid DNA are used.

(d) Sendai and influenza chimerasomes mediate high-efficiency stable gene transfer in animals

Success in achieving chimerasome-mediated stable gene transfer in vitro provided impetus to attempt similar experiments in animals. The proteoliposomes used in this study were large, unilamellar, and contained the glycoproteins from either Sendai or influenza virus integrated in the lipid bilayer. The proteoliposomes were made by the DC protein-cochleate procedure, using rotary dialysis at the vesicle formation step (Gould-Fogerite and Mannino, 1985; Gould-Fogerite et al., 1988).

The DNA transferred was a plasmid which, in animal cells, expresses the polyoma virus early genes: large T, middle T, and small T (Zhu et al., 1984; Triesman et al., 1986). Expression of these genes can cause transformation of mouse cells in vitro and in vivo (reviewed in Tooze, 1980). Virus is not produced, because the viral late (structural) genes are interrupted with bacterial plasmid sequences (Zhu et al., 1984; Triesman et al., 1981). The plasmid does not replicate in eukaryotic cell nuclei, due to the presence of a *Xho*I linker in the PyV *ori*. PyV-based DNA, containing a functional origin of replication and expressing the large-T antigen, tends to be lost from cells due to recombination out of the chromosome, producing circular pieces of DNA (Pellegrini et al., 1984). Therefore, a non-functional PyV *ori* was used to favor the stable presence of the DNA after chromosomal integration.

Chimerasomes were injected subcutaneously in the suprascapular region of two-day-old AKR mice (Table II). Large, rapidly growing tumors developed on the backs or flanks of some mice at six to seven months of age. Microscopic examination of fixed tissue revealed that most tumors were poorly differentiated fibrosarcomas. No tumors were observed when buffer-containing proteoliposomes, alone or with 5.0 μg of unencapsulated DNA, were injected.

Tumor tissue or subcutaneous tissue from the

TABLE II

Stable gene transfer and expression in mice mediated by chimerasomes[a]

	Encapsulated DNA (μg)	Exterior DNA (μg)	Number of mice with tumors[d]
Chimerasomes[b]			
Sendai	5.0	0	3/6
Sendai	0.5	0	1/6
Influenza	5.0	0	1/5
Influenza	0.5	0	0/5
Control proteoliposomes[c]			
Sendai	0	5.0	0/7
Influenza	0	5.0	0/3
Sendai	0	0	0/5

[a] Chimerasomes containing a plasmid encoding the polyoma-virus-transforming genes, but which does not produce virus, were injected subcutaneously into mice. Plasmid p48.19 (a generous gift of Gertruida Veldman, Genetics Institute, Boston, MA) contains the same polyoma virus sequences as p53.A6.6 (Zhu et al., 1984; Triesman et al., 1981-ATCC No. 45017). The early region is intact. The viral late region is interrupted at the *Bam*HI site by *Bam*HI-linearized *E. coli* pxf3 (Hanahan, 1983). The polyoma origin of replication was inactivated by insertion of a *Xho*I linker between nt 35 and 37. Samples, (0.1 ml) of chimerasomes or buffer-filled proteoliposomes alone, or mixed with unencapsulated DNA, were injected subcutaneously in the suprascapular region of two-day-old AKR mice.

[b] Chimerasomes were prepared by encapsulating purified plasmid in proteoliposomes, containing the lipid-bilayer-integrated envelope glycoproteins of either Sendai or influenza virus by the DC protein-cochleate method, using rotary dialysis at the vesicle formation step (Gould-Fogerite and Mannino, 1985; Gould-Fogerite et al., 1988).

[c] Buffer-filled proteoliposomes alone or mixed with unencapsulated plasmid DNA, were injected.

[d] Tumors developed on the back or sides of some mice at six to seven months of age. All mice were killed at eight months of age. Results were expressed as a fraction of the number of mice in a group.

region of either the chimerasome or proteoliposome injection were grown in tissue culture. The cultured cells were then analyzed for the presence of proteins encoded by the injected DNA. Cultured cells from all tumors were positive when stained with PyV-tumor antiserum (not shown). This antiserum, directed primarily against PyV large-T, showed mainly nuclear and some membrane staining. The tumors were also positive when stained with monoclonal antibodies directed against PyV middle-T, which has cytoplasmic, perinuclear and membrane distribution (not shown). In addition, subcutaneous tissue from animals bearing tumors, although not part of or contiguous with the tumors, was also strongly positive for PyV early proteins. In contrast, all cells from subcutaneous tissue from animals that received buffer-filled proteoliposomes or buffer-filled proteoliposomes with unencapsulated DNA were negative when stained with the same antisera.

Cell lines (not clonally derived) were obtained from most of the tumors and from several subcutaneous samples from tumor-bearing mice. Southern-blot analysis was performed on chromosomal DNA extracted from several of these cell lines (not shown). The DNA was cleaved with *Eco*RI and *Xho*I endonucleases and probed with nick-translated [^{32}P]plasmid. All cell lines showed a positive signal with fragments of the sizes predicted from the restriction map of the chimerasome-encapsulated plasmid, as well as additional fragments.

Attempts to establish cell lines from subcutaneous tissue from the region of chimerasome injection in animals which did not develop tumors, were unsuccessful. Some cells were derived from explants taken from the site of injection from several animals. These cells were negative when stained for polyoma early antigens.

Five out of 22 (23%) mice injected with chimerasomes developed tumors. Efficiency of tumor formation was dependent on the quantity of chimerasome-encapsulated DNA injected. Four out of 11 (36%) mice receiving the 5.0-μg dose of DNA, vs. one of eleven (9%) receiving the 0.5-μg dose, developed tumors. Injection of these quantities of chimerasomes did not result in any adverse effects, indicating that larger dosages may be used, possibly leading to even higher efficiencies of gene transfer.

Sendai chimerasomes were more effective than

influenza chimerasomes at gene transfer in vivo. Three of six (50%) mice receiving the higher dose of Sendai chimerasomes (approx. 5 μg of encapsulated DNA injected per mouse), developed tumors. Four of twelve mice (33%), which were injected with Sendai chimerasomes, vs. one out of ten mice (10%) injected with influenza chimerasomes, developed tumors. Sendai proteoliposomes, like native virions, fuse at the surface of cells at neutral pH. In contrast, in vivo, influenza proteoliposomes (and viruses) require exposure to low pH in an endocytic vesicle to mediate membrane fusion. Only a small proportion of the proteoliposomes generated by the protein-cochleate method are small enough to be endocytosed by most cell types. This may account for the difference in gene transfer efficiency observed in vivo between Sendai chimerasomes and influenza chimerasomes.

No mice receiving either empty proteoliposomes with unencapsulated DNA (ten mice), or empty proteoliposomes alone (five mice), developed clearly identifiable tumors. Histologic evaluation of tissue at the site of injection revealed a small area of undifferentiated fibrosarcoma surrounded by fatty tissue in one animal which had been injected with proteoliposomes and unencapsulated DNA. We were unable to culture cells from this specimen and were therefore unable to analyze it for the presence of PyV DNA or its products. We cannot exclude the possibility that gene transfer took place. In vivo gene transfer by injection of DNA has been reported (Asselin et al., 1983). Indeed, co-injection of unencapsulated DNA with fusogenic proteoliposomes may have facilitated its entry into cells.

It is possible that the efficiency of gene transfer is not truly reflected in this assay system. PyV-transformed cells are highly antigenic and can be removed by the immune system (Defendi and Lehman, 1965). AKR mice develop lymphomas at six to nine months of age, at which time their immunity becomes impaired. It is interesting that overt tumor development coincided with the onset of immune disorders in these mice.

The results obtained from this initial study are very encouraging as to the applicability of these delivery vesicles to in vivo gene transfer. We are in the process of developing this into a cell type-specific delivery system, with special interest in targeting to transformed, infected, and genetically deficient cells.

(e) Conclusi ns

(1) A highly effective delivery system for animal cells is produced when the envelope glycoproteins from Sendai or influenza virus are reconstituted using the protein-cochleate method (Gould-Fogerite and Mannino, 1985; Gould-Fogerite et al., 1988).

(2) This proteoliposome system has flexibility in the choice of viral glycoproteins and in the proteoliposome formation procedure itself. Delivery can be gradual, at neutral pH, when Sendai vesicles are used, or synchronous, by low pH-activated fusion of influenza vesicles.

(3) Injection of Sendai or influenza chimerasomes containing encapsulated plasmid DNA subcutaneously into the suprascapular region of two-day-old mice led to stable gene transfer and expression.

(4) Proteoliposomes can be used to deliver a wide variety of large and small molecules to the cytoplasm of entire populations of cells in culture. The ability to deliver DNA, proteins and small molecules to large numbers of cells should facilitate many studies which would be difficult or not feasible to perform using other currently available cellular delivery techniques.

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